Supporting Information

A rapid evaluation method for the quality consistency assessment and spectrum-effect relationship study of Xiaohuoluo Pills developed based on the spectral and chromatography combined technology

Fangfang Cheng[1,2#], Yiting Gao[2#], Beibei Ren[3#], Zhongyang Zhang[2], Minghui Dong[2], Guoxiang Sun[4], Chaohui Song[1,2], Siqi Wang[4], Haixia Gao[2], Chunhua Zhou[1], Lingjiao Wang[1]*

[1] Department of Clinical Pharmacy, the First Hospital of Hebei Medical University, Shijiazhuang, Hebei, China.

[2] Hebei Medical University, Shijiazhuang, Hebei, China.

[3] Hebei Provincial Center for Disease Control and Prevention, Shijiazhuang, Hebei, China.

[4] School of Pharmacy, Shenyang Pharmaceutical University, Shenyang, China.

Correspondence

Lingjiao Wang, Department of Clinical Pharmacy, The First Hospital of Hebei Medical University, Shijiazhuang, Hebei Province, China

* Corresponding author:

Lingjiao Wang: wlj@hebmu.edu.cn

Haixia Gao: gaohx686@hebmu.edu.cn

Chunhua Zhou: zhouchunhua80@126.com

# These authors have contributed equally to this work: Fangfang Cheng, Yiting Gao and Beibei Ren contributed equally to this work.

1. Materials and Methods

1.1 Chemicals and Reagents

XHLPs samples were provided by Shanghai Huayuan Pharmaceutical Anhui Plant Pharmaceutical Industry Co.,Ltd (numbered S1-S18) and Shandong Xindalu Pharmaceutical Co.,Ltd. (S1-S21) manufacturers, HPLC grade methanol and acetonitrilewere purchased from Thermo Fisher Scientific (USA) and Supelco (USA). Phosphoric acid (analytical grade) is sourced from Xilong Chemical Co., Ltd. (Guangdong, China). Watson water（A.S.WATSON TM LIMITED, China) was used throughout the test. Potassium bromide (KBr) for spectroscopy (> 99.0 %) was purchased from Pinchuang Technology Development Co., Ltd. (Tianjin,China). Gallic Acid(GA, purity98%, BatchNO. DSTDM000802), Benzoylmesaconine (BLA, purity 98%, Batch NO. DST23022-056) and Benzoylaconine (BA, purity 98%, Batch NO. DSTDB005502) were purchased from Chengdu Lemeitian Pharmaceutical Technology Co., Ltd(Sichuan, China). DPPH (98%) was sourced from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China).

1.2 Sample and standard solution preparation

Three reference standards (GA, BLA, and BA) were accurately weighed, dissolved in methanol-water (80:20, v/v), and stored at 4°C for later use. The above standard solution was diluted with methanol-water (80:20, v/v) to obtain the necessary concentration range, resulting in at least 6 concentration levels of standard curves. 21 batches of XHLPs were uniformly pulverized. Exactly 1g of sample powder was weighed, placed into a 15ml centrifuge tube, and then 8mL of methanol-water (80:20, v/v) was added. Ultrasonic extraction was carried out at 45°C for 20 minutes, followed by centrifugation at room temperature at 10000r/min and 80Hz for 10 minutes. The supernatant was filtered using a 0.22mm filter membrane for analysis or further dilution as the original solution for antioxidant determination.

In the infrared experiment accurately weigh 6 mg of the sample and 150 mg of potassium bromide (KBr) crystal. Place them carefully and evenly in an agate mortar,

take 100 mg mixture and press for 1 min at 15 MPa to confirm that they mix into a sheet.

1.3 Theory of CQRFM

In order to reduce errors caused by fingerprints and strengthen the control over qualitative and quantitative similarities, we use CQRFM to perform overall quantitative analysis of system fingerprints based on macro qualitative analysis combined with a simple quantification ratio fingerprint method[1]. CQRFM is based on two indicators: macro qualitative similarity (S_r) and macro quantitative similarity (P_r), which retain the performance of extreme ratio quantification fingerprinting and simple ratio quantification fingerprinting, to a certain extent eliminating the large errors caused by large ratio fingerprints, while enhancing control over qualitative and quantitative parameters[2]. S_r calculated by Eq.1 is used to monitor the category properties of ratio fingerprints, while P_r obtained from Eq.3 is used to monitor the overall quantitative properties of ratio fingerprints.

The peak areas of the sample fingerprint (SFP) and the reference fingerprint (RFP) are denoted as x_i and y_i, respectively. The SFP vector is calculated as $\overrightarrow{X_R}=(x_1/y_1,x_2/y_2,...,x_n/y_n)=(r_1,r_2,...r_n)$, where r_i represents the weight ratio of each fingerprint. It is constrained that $0 \leq R_i < 2$, which helps reduce the differences between the peaks. The RFP vector is represented as $\overrightarrow{Y_R}=(1,1,...,1)=(100\%, 100\%, ..., 100\%)$, indicating that the content of each fingerprint is 1 (100%).

S_F represents qualitative similarity and is mainly influenced by large peaks, making it difficult to reflect small peaks and easily causing peak loss. S'_F represents a modified qualitative similarity that is insensitive to changes in large peaks. In this case, the angular cosine S_r between $\overrightarrow{X_R}$ and $\overrightarrow{Y_R}$ is calculated as the limit ratio qualitative similarity, which allows the peak areas of different fingerprints to compensate for each other, eliminating the phenomenon of large peaks masking small peaks. The control of SFP is normalized with the weighted RFP, and the introduction of R (Eq.2) as macroscopic content similarity is incorporated into the calculation to correct and

obtain P_r.

$$S_r = \frac{1}{2}(S'_F + \frac{S_F + S'_r}{2})$$

$$= \frac{1}{2}\frac{\sum_{i=1}^{n} x_i y_i}{\sqrt{\sum_{i=1}^{n} x_i^2}\sqrt{\sum_{i=1}^{n} y_i^2}} + \frac{1}{2}\frac{\sum_{i=1}^{n}\frac{x_i}{y_i}}{\sqrt{\sum_{i=1}^{n}\frac{x_i}{y_i}^2}} + \frac{\sum_{i=1}^{n} r_i}{\sqrt{n\sum_{i=1}^{n} r_i^2}} \qquad \text{Eq.1}$$

$$R = \sum_{i=1}^{n} q_i r_i \times 100\% = \sum_{i=1}^{n}\frac{y_i}{\sum_{i=1}^{n} y_i} r_i \times 100\% \qquad \text{Eq.2}$$

$$P_r = R\frac{\sum_{i=1}^{n} x_i y_i}{2\sqrt{\sum_{i=1}^{n} x_i^2}\sqrt{\sum_{i=1}^{n} y_i^2}} + \frac{1}{4} r + \frac{\sum_{i=1}^{n} r_i}{\sqrt{n\sum_{i=1}^{n} r_i^2}} \qquad \text{Eq.3}$$

Table S1 provides the quality evaluation criteria for traditional Chinese medicine based on CQRFM, where the quality can be divided into 8 levels. Ideally, the ideal limit values for qualified samples should be $S_r \geq 0.90$ and $70\% \leq P_r \leq 130\%$. The determination of the final quality level often depends on the lowest level, and therefore, samples with a quality level <5 are generally considered acceptable.

2. Method validation

2.1 FT-IR

Using the potassium bromide pellet method mentioned for FT-IR experiments. To ensure the applicability of fingerprint analysis and reduce random errors, all samples were analyzed consecutively within one day. According to the methodology outlined in section 2.3, the S5 batch samples underwent analysis to validate the method's applicability. The single extraction from each S5 sample underwent six analyses to assess instrument precision. S5 samples were consecutively extracted six times, yielding six homogeneous analytes, these analytes were subsequently subjected to infrared analysis to verify the method's reproducibility. P_r was used as an evaluation parameter for method validation, and the RSDs of P_r were 0.58% and 0.29%

respectively, indicating that the FT-IR spectroscopic method meets the requirements of XHLP fingerprint analysis.

2.2 UV

All samples in the UV experiment were processed according to the procedures outlined in section 2.2, ensuring consecutive analysis within a single day. To verify the applicability of UV, we randomly selected S5 for precision and repeatability testing, the testing method is the same as that for FT-IR analysis,and monitored its compliance using the RSD of sample Pr values. The results showed RSD values of 0.36% and 0.80% respectively, meeting the requirements for fingerprint analysis. Subsequently, we conducted an analysis of the stability of the samples. A stability test was established, at room temperature, the same sample solution was analyzed every 4 hours, with a total of six injections, ensuring a 24-hour interval between the first and last injections, yielding an RSD of 0.31%, indicating stability of the analyte within 24 hours.

2.3 HPLC

To validate the feasibility of the HPLC fingerprinting method, this study randomly selected S5 samples for methodological verification and assessed the precision, repeatability, and stability of the HPLC analysis instrument. Sample processing methods are detailed in Section 2.2. Precision was evaluated by processing one batch sample, dividing the extract into five portions, and continuously injecting these portions. Repeatability was assessed by processing samples from the same batch using the identical method five times, with each analysis continuously injecting the substances obtained. Stability was determined by injecting the processed substance solution once every 5 hours for a total of 5 injections, ensuring a 24-hour interval between the first and last injections. Following the sample analysis, three common peaks were chosen, and their relative retention times and peak areas were utilized to calculate the relative standard deviation (RSD) for variability assessment. The average RSD results for the precision, repeatability, and stability of sample peaks were 1.9%, 0.94%, and 1.30%, respectively, all values being less than 2.00%. These findings affirm the reliability and accuracy of this method for fingerprint analysis

across 21 samples.

In terms of quantitative analysis, various parameters such as precision, repeatability, stability, linearity, detection limit (LOD), quantification limit (LOQ), and instrument recovery rate were assessed to ensure the reliability of the quantitative method. A regression curve was generated by plotting the relationship between the peak area (y) at six points and the concentration (x, mg/mL) of the reference substance in the prepared series of mixed standards. The linear results, LOD, and LOQ for the three markers are presented in Table S2. It was observed that there exists a strong linear correlation ($r \geq 0.999$) between the peak area and component concentration within the desired range. It is noteworthy that the average recovery rates for GA, BLA, and BA were 107.56%, 98.97%, and 105.46%, respectively, all of which had RSD values below 2%. Consequently, the verification results affirm the feasibility, applicability, and accuracy of the quantitative method in determining the content of the three designated standard compounds.

---

1 Sun, G. X., Hou, Z. F., Bi, Y. M., Bi, K. S., and Sun, Y. Q., *Acta Pharm. Sin.,* 2006, 41(9), 857–862.

2 Sun, G.X., Wu, Y., Liu, Z.B., Li, Y.F., Guo, Y., *Anal. Methods,* 2014, 6, 838–849.

Table S1. TCM quality grade divided by CQRFM.

Grade	1	2	3	4	5	6	7	8
Sr	≥0.95	≥0.9	≥0.85	≥0.80	≥0.70	≥0.60	≥0.50	≤0.5
Pr	95-105	90-110	85-115	80-120	70-130	60-140	50-150	0-∞
α	≤0.05	≤0.10	≤0.15	≤0.20	≤0.30	≤0.40	≤0.50	＞0.50
Quality	Best	Better	Good	Find	Moderate	Common	Defective	Inferior

Table.S2 The linear regression equations, r, LOD, LOQ and linear range for GA, BLA, and BA.

Compound	Linear regression	r	Linear range(ug/ml)	LOD(ng)	LOQ(ng)
GA	y = 7404.4x - 9532.9	1	15.625-500	1342	1469
BLA	y = 10749x + 51.383	1	0.3125-10	32.90	120.8
BA	y = 16470x + 285.87	1	0.3125-10	7.23	64.61

Table. S3 The antioxidant results of 21 batches of samples.

No.	Linear equation	IC_{50}(mg/ml)	R^2
S1	y = 0.079x - 0.0342	6.762	0.99
S2	y = 0.0859x - 0.0832	6.789	0.9945
S3	y = 0.0616x + 0.0247	7.716	0.9944
S4	y = 0.0795x - 0.0323	6.696	0.9998
S5	y = 0.0589x + 0.0439	7.744	0.9913
S6	y = 0.0664x - 0.0236	7.886	0.9924
S7	y = 0.0748x + 0.0047	6.622	0.9971
S8	y = 0.0808x + 0.0384	5.713	0.9998
S9	y = 0.0661x - 0.0033	7.614	0.99688
S10	y = 0.0447x + 0.0539	9.980	0.9956
S11	y = 0.0764x + 0.0075	6.445	0.9998
S12	y = 0.0643x + 0.0086	7.642	0.9976
S13	y = 0.0674x + 0.0228	7.080	0.9948
S14	y = 0.076x + 0.0286	6.203	0.9901
S15	y = 0.0737x - 0.0717	7.757	0.9966
S16	y = 0.0856x + 0.0427	5.342	0.9924
S17	y = 0.0696x + 0.0636	6.270	0.9984
S18	y = 0.065x + 0.0929	6.263	0.9903
S19	y = 0.0289x + 0.015	16.782	0.9918
S20	y = 0.0202x + 0.017	23.911	0.9936
S21	y = 0.0172x + 0.0064	28.698	0.9920

Table.S4 The relevant parameters of the validation set and Training set for three spectral graphs in the OPLS model.

		R^2	R^2Y	Q^2	RMSEE	RMSEcv	RMSEP
FT-IR	validation set	0.9023	0.9910	0.8470	0.8782	2.5650	2.0261
	Training set	0.9910					
UV	validation set	0.8828	0.9990	0.8880	0.2401	2.1981	4.9804
	Training set	0.9992					
HPLC	validation set	0.9418	0.9970	0.9830	1.1835	2.1098	5.3069
	Training set	0.9736					